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14. ABSTRACT

This proposal attempted to identify drugs for treating metastatic melanoma by utilizing meta-analysis of melanoma transcriptome data to generate a metastatic melanoma gene signature and apply this gene signature to the Connectivity Map database (C-Map) of drug gene signatures. Results from our study provide strong support that transcriptome analysis can prioritize drugs that may be effective against metastatic melanoma. We generated a metastatic melanoma-specific gene signature by selecting the genes that are commonly perturbed in metastatic melanoma. We showed that the C-Map database of FDA approved and other small molecule drugs can be used to select candidate anti-melanoma drugs that can be evaluated in cell cultures of metastatic melanoma cells and eventually in xenograft models for preclinical validation. We identified several drugs that are predicted to reverse the metastatic phenotype of melanoma and our results indicate that 4 out of 9 drugs, selected as high scorers in the C-Map analysis of metastatic melanoma, are in fact strong inducers of apoptosis in melanoma cell lines, demonstrating that these unrelated drugs may indeed lead to anti-melanoma efficacy. These drugs will be tested in xenograft experiments in mice. Demonstrated anti-tumor efficacy would be key for advancing a drug for human clinical testing.

15. SUBJECT TERMS
Meta-analysis of genomics data, Connectivity Map Database, Metastatic melanoma, Metastatic melanoma gene signature, Drug gene signatures, Melanoma gene signatures reversing drug signatures, Candidate small molecule compounds targeting metastatic melanoma, Apoptosis induction, Melanoma xenografts

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Introduction:

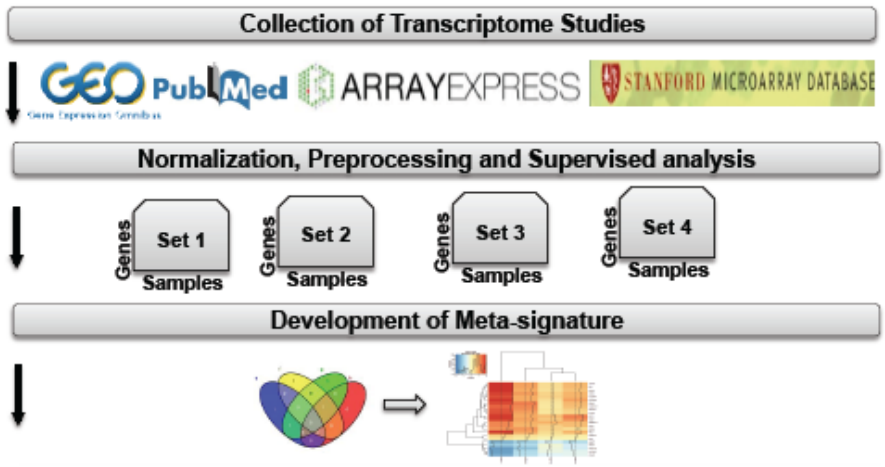
Project Title: Testing New Drugs for treatment of Melanoma Patients Applying Connectivity Map Database Analysis with Melanoma Gene Signatures

Despite some recent advances in therapeutic management of patients with metastatic melanoma, most patients with advanced melanoma succumb to the disease. In an attempt to remedy this situation, we proposed to use knowledge from melanoma gene profiling studies to identify pathways amenable to “targeted” therapies, namely advanced melanoma specific gene signatures with outcome data. We suggested toapply a highly innovative approach to identify and validate new drugs against melanoma. The innovative concept is based on the premise that drugs that revert the poor outcome, metastatic melanoma gene signature towards a “normal melanocyte” or good outcome, early stage melanoma gene signature will have a strong anti-cancer effect. Taking advantage of the Connectivity Map database that contains gene signatures for 1309 drugs we tested this hypothesis with drugs that elicit gene signatures most opposed to advanced melanoma. This therapeutic strategy was anticipated to rationally identify drugs that synergize with current standard-of-care therapy to target advanced melanoma. The drugs with the highest ranking scores of inverted gene signatures were to be tested for their abilities to kill metastatic melanoma cells or to inhibit their invasive activities in culture. The 3 most effective drugs were to be tested in a human xenograft metastatic melanoma animal model for their abilities to inhibit or reduce tumor growth and metastasis. Thus, our aims were: Aim 1. Apply melanoma gene signatures to the CMAP database in order to identify drugs that are expected to reverse melanoma specific gene expression profiles; and, Aim 2. Test the highest scoring drugs from the CMAP analysis in melanoma cell lines and animal models.

<u>Task 1.</u> Apply melanoma gene signatures to the CMAP database in order to identify drugs that are expected to reverse melanoma specific gene expression profiles

1a. Published melanoma transcriptional profiling data including primary and metastatic melanoma as well as outcome related gene signatures will be selected and meta-analysis will be performed to identify a common set of genes that is specific for metastatic melanoma and poor outcome (Metastatic and Poor Outcome melanoma specific gene signature).

The urgent need for improvement in the diagnosis and treatment of metastatic melanoma patients has spurred a number of studies aimed at identifying differentially expressed genes in melanoma by taking advantage of new high-throughput techniques for examining molecular alterations. However, the vast amount of microarray gene expression data has not yet translated into a clinically useful biomarker or improved treatment, although recent progress with targeting mutant BRAF has resulted in major temporary improvements including temporary disappearance of metastases. Various individual transcriptome studies have identified genes that are differentially expressed in melanoma; however, no meta-analysis has been performed to determine how robust and reproducible these profiling data are and whether a specific set of genes is differentially expressed in metastases that could be further explored for therapeutic development.



One option to overcome the limitations of individual microarray analysis is meta-analysis of multiple gene expression profiling studies: due to increased statistical power this approach may identify a more reliable genetic signature by detecting potentially important genes missed in a single study and eliminating possible false positives. However, the lack of concordance of gene signature lists in published reports due to variations in microarray platforms, techniques and analysis methods makes comparative analysis of individual microarray studies using standard approaches difficult. Furthermore, meta-analysis approaches that incorporate reanalysis of original data from multiple microarray studies (as opposed to the conventional approach of comparing the interpreted results of separate studies) generate gene signatures with increased reproducibility and sensitivity, thus revealing biological insight not evident in individual datasets. Fortunately, meta-analysis approaches that incorporate re-analysis of the original data can generate gene signatures with increased reproducibility and sensitivity, thus revealing biological insight that was not evident in the individual datasets. We hypothesized that a robust meta-analysis strategy to combine multiple microarray datasets will uncover a robust gene expression signature for metastatic melanoma that can be exploited to discover and repurpose drugs specific for metastatic melanoma. To discover a metastatic melanoma gene signature we distilled the plethora of potential melanoma gene expression changes through an

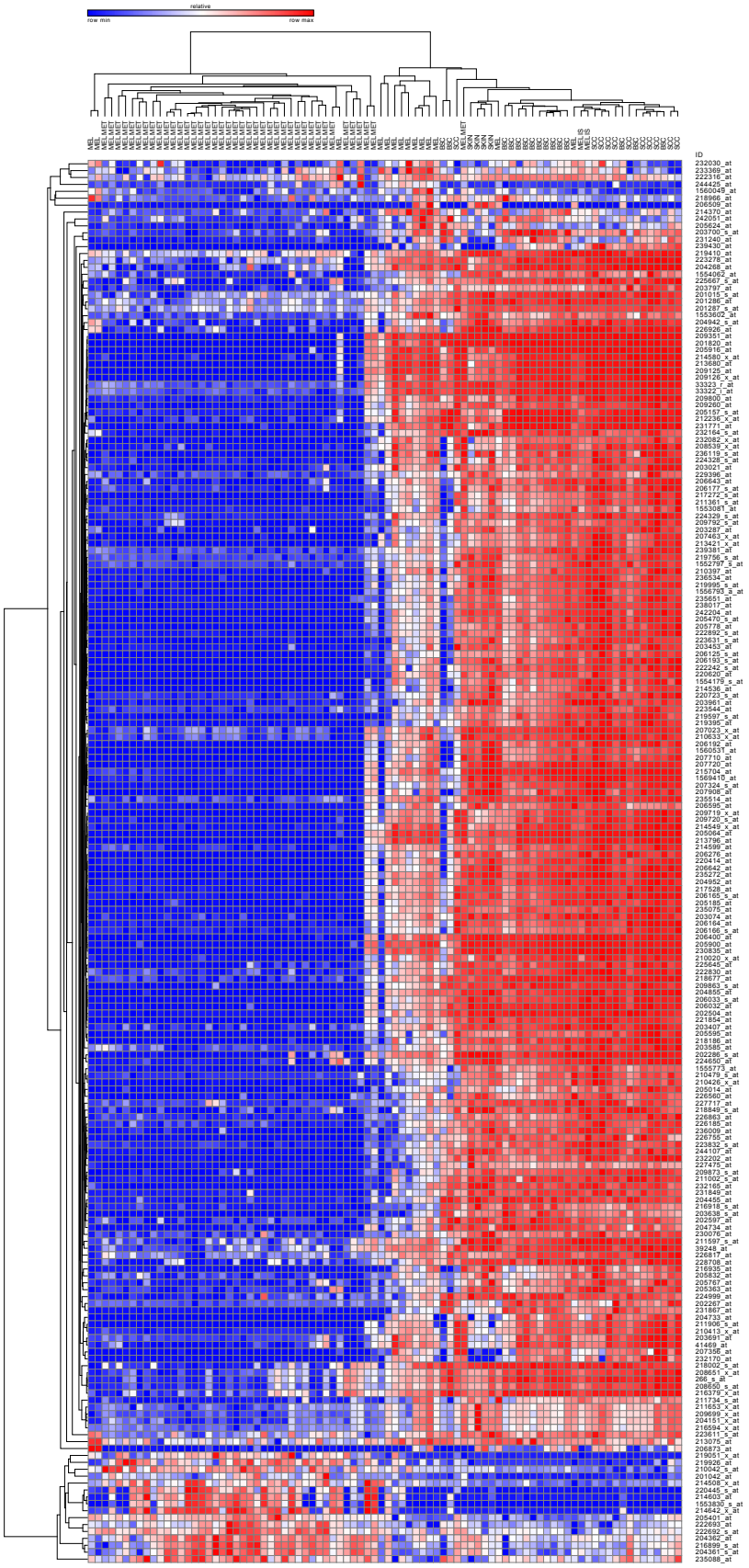


Fig. 1. Hierarchical clustering of GSE7553 metastatic gene signature.
Metastatic melanoma samples are in the left branch of the tree and the primary melanoma and other skin cancers are on the right branch.

optimized meta-analysis strategy that included normalization and statistical testing procedures on the raw data from three independent melanoma transcriptome datasets that contained metastatic melanomas as well as separate analyses on the individual datasets using identical analysis parameters.

Extensive search of the literature and publicly available microarray repositories identified three gene expression studies of human patient melanoma specimens that included metastatic samples. We collected these 3 datasets that include samples of normal skin, basal and squamous carcinoma, primary melanoma, and metastatic melanoma for development of a metastatic melanoma gene signature. All datasets utilized Affymetrix GeneChip arrays. The GEO datasets include i) GSE7553: 15 basal cell carcinomas, 2 melanomas in situ, 14 primary melanomas, 40 metastatic melanomas, 5 normal skins, 11 squamous cell carcinomas; Affymetrix Human Genome U133 Plus 2.0 Array; ii) GSE8401: 31 primary melanomas and 52 metastatic melanomas; Affymetrix Human Genome U133A Array; iii) GSE15605: 46 primary melanomas, 12 regional and distal metastases, 16 normal skins; Affymetrix Human Genome U133 Plus 2.0 Array.

Data analysis was executed through the use of R packages implemented in Bioconductor. Each dataset was normalized from raw data using the Frozen RMA (fRMA) algorithm. We applied the same global normalization and filtering approach to all datasets and implemented a consistent procedure for differential gene expression analysis across all datasets to minimize bias introduced by different laboratories and different types of arrays. Z-statistics normalization preprocessed the normalized datasets to reduce batch effects among different datasets. Differentially expressed genes were identified by empirical Bayes moderated t-statistic. We selected differentially expressed genes using a very stringent Benjamini and Hochberg method for multiple test correction to control for false discovery rate (FDR). The comparative reanalysis of raw expression data from the three independent melanoma transcriptional profiling with the empirical Bayes approach and using identical normalization and statistical methods for each database resulted in three lists of differentially expressed genes. Figure 1 shows an example of the metastasis-specific genes for GSE7553. Hierarchical clustering utilizing the top 200 genes accurately separates the metastatic melanoma samples from normal skin, basal cell carcinoma, squamous cell carcinoma, and primary melanoma with only one metastatic melanoma and 3 primary melanoma clustering in the wrong group. Similar results were obtained for the other datasets.

Generation of the metastatic melanoma gene signature for the C-MAP analysis

We selected the top ranking genes from the list of differentially expressed genes for each dataset that discriminate between metastatic melanoma and primary melanoma, other skin cancers and normal skin: selection was based on each gene being differentially expressed in at least 2 of 3 microarray studies, with a FDR of <10%. Statistical significance comparison for each gene across all studies identified genes consistently differentially expressed in metastatic melanoma compared to primary melanoma, other skin cancers and normal skin. 203 genes common with concordant directionality in at least 2 of the 3 datasets were identified by Venn diagram analysis of the three sets of differentially expressed genes which were used for further Connectivity Map database analysis.

We spent significantly more time on this task than anticipated, because development of the specific algorithms and fine tuning of the analytic strategy to accomplish this task was not immediately straightforward. We did not generate a poor outcome gene signature due to lack of enough datasets and an overall strong overlap between metastasis and poor outcome.

1b. The melanoma specific gene signature established in Task 1a will be screened against 7000 gene signatures for 1309 bioactive small molecules in the Connectivity Map database in order to identify and rank small molecules that reverse the Metastatic and Poor Outcome melanoma specific gene signature.

TABLE 1

Compounds	Enrichmen
MS-275	-0.999
cantharidin	-0.994
dexverapamil	-0.991
exemestane	-0.963
PHA-00665752	-0.962
meptazinol	-0.937
celastrol	-0.923
quinostatin	-0.895
piperlongumine	-0.877
difenidol	-0.873
molindone	-0.871
Prestwick-1084	-0.869
omeprazole	-0.867
trazodone	-0.867
1,4-chrysenequinone	-0.853
5252917	-0.842
piperidolate	-0.825
5114445	-0.825
Prestwick-559	-0.822
DL-thiorphan	-0.821
liothyronine	-0.805
5109870	-0.8
CAY-10397	-0.798
blebbistatin	-0.795
chrysin	-0.79
corynanthine	-0.789
naftopidil	-0.783
pararosaniline	-0.781
metyrapone	-0.779
sanguinarine	-0.769
hexestrol	-0.762
cetirizine	-0.759
latamoxef	-0.752
demecolcine	-0.752
perhexiline	-0.739
sulconazole	-0.739
5152487	-0.739
glafenine	-0.738
5255229	-0.728
phthalylsulfathiazole	-0.727
terfenadine	-0.724
tyloxapol	-0.723
cortisone	-0.718
ebselen	-0.717
5248896	-0.717
flunarizine	-0.715
thioguanosine	-0.715
ifenprodil	-0.715
betahistine	-0.708
doxylamine	-0.703

Discovery of candidate therapeutic agents in the C-Map database that are anticipated to reverse the metastatic melanoma gene signature

We applied the 203 gene metastatic melanoma expression signature to the C-Map database using gene set enrichment to identify drugs that were counter-acting the metastatic melanoma-specific signature. C-Map database analysis identified small molecules that were anti-correlated with the metastatic melanoma-specific signature. These compounds were ranked according to their inverse geneset enrichment score. The top 50 drugs are shown in Table 1.

1c. The 10 small molecules with the highest ranking scores of inverted gene signatures will be selected for further evaluation and validation in *in vitro* and *in vivo* assays. Primary focus for this proposal will be on FDA approved drugs.

We selected only the highest ranking compounds that are FDA approved drugs for further evaluation of potential therapeutic efficacy against melanoma cells, with the assumption that if we can demonstrate efficacy of a FDA approved drug, clinical translation would be much faster than if we focus on experimental small molecules that are not approved for human use. We chose the following 9 drugs (highlighted in Table 1) for further evaluation: MS-275, cantharidin, dexverapamil, exemestane, meptazinol, trazodone, naftopitil, metyrapone, cetirizine.

<u>Task 2.</u> Test the highest scoring drugs from the CMAP analysis in melanoma cell lines and animal models

2a. The 10 highest scoring FDA approved small molecule drugs from Task 1 at physiologically achievable doses will be tested in metastatic melanoma cell lines (A375M, R-18, SK-MEL-28, MALME-3M) for their abilities to inhibit cell proliferation, migration, invasion, and soft agar anchorage-independent growth and to induce cell cycle arrest or apoptosis using high throughput liquid handling robots and 96 well plates.

Four of the nine high scoring candidate drugs induce apoptosis in melanoma cell lines

We evaluated nine of the high scoring drugs for metastatic melanoma with regard to their abilities to induce apoptosis in the metastatic melanoma cell line, A375M. Four (MS-275, dexverapamil, naftopidil, exemestane) out of the nine tested drugs strongly induced cell death relative to the solvent DMSO when used at the concentration applied in the C-Map database demonstrating that these four structurally unrelated drugs indeed induce anti-melanoma activity. The dose response for each of the drugs was determined using a dose response curve in these cells for

the 4 apoptosis inducing drugs (Fig. 2). Figure 2 demonstrates each of the 4 drugs inducing apoptosis efficiently at physiologically relevant doses.

To determine whether these drugs have similar effects on other metastatic melanoma cell lines, we treated MALM-3M and SK-MEL-28 melanoma cells with the same four drugs. As shown in Figure 3 all four drugs also induced apoptosis in the other two cell lines, although there were some slight differences with regard to sensitivities of the different cell lines to the different drugs.

A375M #	Dose	value
MS-275	50micromolar	**1.271**
MS-275	25micromolar	**0.985**
MS-275	10micromolar	**0.734**
MS-275	5micromolar	**0.551**
Dexverapamil	20micromolar	**0.917**
Dexverapamil	10micromolar	**0.703**
Dexverapamil	4micromolar	**0.624**
Dexverapamil	2micromolar	**0.449**
Naftopidil	10micromolar	**1.002**
Naftopidil	5micromolar	**0.889**
Naftopidil	2micromolar	**0.615**
Naftopidil	1micromolar	**0.338**
Exemestane	10 nanomolar	**0.835**
Exemestane	5 nanomolar	**0.653**
Exemestane	2 nanomolar	**0.526**
Exemestane	1 nanomolar	**0.431**
DMSO	0.50%	**0.345**

Fig. 2. Apoptosis assay for A375M cells treated with the indicated doses of drugs that scored high on the Connectivity Map analysis.

Drug	Dose	MALM-3M Apoptosis	std dev	SK-MEL-28 Apoptosis	std dev
MS-275	50μM	**1.038**	0.033	**1.426**	0.042
Dexverapamil	20μM	**0.721**	0.021	**0.898**	0.017
Naftopidil	10μM	**1.211**	0.037	**0.932**	0.064

Fig. 3. Apoptosis assay for MALM-3M and SK-MEL-28 cells, treated with the indicated doses of drugs that scored high on the Connectivity Map analysis and induced apoptosis in A375M cells.

2b. The 4 most effective drugs will also be tested for synergistic activity in above assays in a dose response curve.

Double and triple combinations of low doses of high scoring candidate drugs are highly effective in inducing apoptosis in melanoma cells

To evaluate whether similar pro-apoptotic activity in metastatic melanoma cell lines could be achieved by combining multiple drugs at low doses that by themselves would not be expected to induce significant apoptosis, we combined the 4 top scoring drugs at doses low enough to not significantly induce apoptosis. As seen in Figure 4, several combinations demonstrated high efficacy in eliciting apoptosis in A375M cells. Specifically, combinations of low doses of MS-275 with naftopidil enhanced apoptosis significantly. Moreover, triple combinations of low doses of the top 4 drugs induced apoptosis more effectively than either alone or double combinations

	A375M
MS-275	0.518
Dexverapamil	0.421
Naftopidil	0.347
Exemestane	0.469
MS-275 + Dexverapamil	0.698
MS-275 + Naftopidil	1.180
MS-275 + Exemestane	0.944
Dexverapamil + Naftopidil	0.735
Dexverapamil + Exemestane	0.632
Naftopidil + Exemestane	0.926
MS-275 + Dexverapamil + Naftopidil	1.298
MS-275 + Dexverapamil + Exemestane	1.141
MS-275 + Naftopidil + Exemestane	1.693
Dexverapamil + Naftopidil + Exemestane	1.170
DMSO	0.329

Fig. 4. Combinations of low doses of 4 Top Scoring Drugs are effective in inducing apoptosis.

demonstrating synergistic activity (Fig. 4).

2c. Seek approval for the animal use including IACUC, DoD Office of Research Protection, Animal Care and Use Review Office (ACURO).

We wrote the animal protocol, submitted it and got approval for protocol #105-2010 on June 9, 2011.

2d. For these proof-of-concept studies anti-tumor and anti-metastatic efficacy of the 3 lead compounds against melanoma, namely compounds that elicit the strongest anti-tumor and/or anti-metastatic efficacy in the cell-based assays, will be assessed in two human melanoma xenograft mouse models (25 mice per study, 75 mice total) using established efficacious treatment doses for these FDA approved drugs. 10^6 A375M and SK-MEL-28 cells, engineered to express firefly luciferase using a retrovirus that encodes a fusion of luciferase and neomycin phosphotransferase (LucNeo), will be implanted and followed for tumor growth and metastasis using *in vivo* bioluminescence imaging (BLI) endpoints. Animals with established tumors will be divided into 3 cohorts to be treated with vehicle, drug at maximally tolerated dose (MTD), and drug at 1/2 x MTD. Animals will be treated for 2-3 weeks, until control arms begin to reach tumor size limits (2000 mm3), or animals become moribund, at which point animals will be humanely euthanized. Primary endpoint is tumor growth and number of metastases, as determined by BLI, and tumor volume measurements. Time-to-sacrifice will be analyzed by log-rank test as secondary endpoint.

We have generated A375M and SK-MEL-28 cells expressing firefly luciferase using a retrovirus that encodes a fusion of luciferase and neomycin phosphotransferase (LucNeo) to develop metastatic melanoma xenograft models. Luciferase expression within the melanoma cells allows *in vivo* imaging for measuring tumor burden and metastasis quantitatively rather than caliper measurements. However, in some other tumor models we have seen drug effects on luciferase activatity which may potentially limit the use of this in vivo imaging strategy. A375M and SK-MEL-28 cells were infected with a VSVG-pseudotyped retrovirus encoding a fusion of firefly luciferase with neomycin phosphotransferase (LucNeo). A375M and SK-MEL-28-LucNeo cells were enriched in 1 mg/ml of G418 and shown to express luciferase.

The generation and selection of the engineered cells did not progress as rapidly as hoped for due to some contamination issues and slow growth of one of the cell lines after G418 selection took significantly longer and as a result more resources than originally anticipated were used. As a result we have not been able to start the xenograft experiments prior to the end of the funding period. Nevertheless, we plan with new funding to implant the A375M and SK-MEL-28-LucNeo cells and follow their tumor growth and metastasis using *in vivo* bioluminescence imaging (BLI) endpoints. Animals with established tumors of ~200 mm3 will be divided into 3 cohorts to be treated with vehicle, MS-275, naftopidil,and exemestane at maximally tolerated dose (MTD), and at ½ x MTD for 2-3 weeks as detailed in our SOW.

2e. If *in vitro* testing identifies combinations of drugs with synergistic activity, the most effective combination will also be tested *in vivo* as above. Animals will be divided into cohorts to be treated with vehicle, each drug individually, and in combination. For this 4-arm study, drugs will be dosed at 1/2 x MTD, to decrease likelihood of combinatorial toxicity in the combination arm.

This task was not initiated due to the aforementioned time constraints.

2f. The same 3 drugs will be tested in the BRAF/PTEN melanoma model using the same study design and endpoints as in task 2b. Tumor burden and number of metastases assessed by BLI will be the primary endpoint, and time-to-sacrifice the secondary endpoint.

This task was not initiated as we used resources and time to pursue and finish task 1 and tasks 2a, 2b and 2c as well as the first part of 2d.

2g. Analyze all xenograft data using thorough statistical approaches and prepare report at the end of performance period.

This task was not initiated as we used resources and time to pursue and finish task 1 and tasks 2a, 2b and 2c as well as the first part of 2d.

Key Research Accomplishments:

- Meta-analysis of 3 metastatic melanoma gene signatures
- Generation of lists of differentially expressed genes between metastatic melanoma and primary melanoma, other skin cancers and normal skin
- Identification of a set of key genes commonly altered in metastatic melanoma
- Identification of candidate small molecule compounds that are anticipated to reverse the metastatic melanoma gene signature
- Selection of 9 high ranking FDA-approved drugs that are predicted to counteract melanoma metastasis pathways
- Validation that 4 of 9 drugs induce cell death efficiently in three metastatic melanoma cell lines
- Synergistic and enhanced melanoma killing by double and triple combinations of low doses of selected combinations of the 4 drugs
- Generation of stable luciferase expressing A375M and SK-MEL-28 cells for the planned
  xenograft studies.

Reportable Outcomes:

Gene signatures of melanoma metastasis-associated genes for 3 published datasets

Gene signatures of melanoma metastasis-associated genes commonly deregulated in 3 published datasets

List of high ranking small molecule drugs expected to reverse the melanoma metastasis gene signature

Apoptosis induction of several metastatic melanoma cell lines by 4 selected FDA approved drugs

Development of luciferase expressing A375M and SK-MEL-28 melanoma cells

Conclusions: The described results provide strong support that transcriptome analysis can enable prediction for drugs that may be effective in inducing of apoptosis in metastatic melanoma cells. We generated a metastatic melanoma-specific gene signature by selecting the genes that are commonly perturbed in at least 3 published datasets of metastatic melanoma. We now show that the Connectivity Map database of FDA approved and other small molecule drugs can be used to select candidate anti-melanoma drugs that can be evaluated in cell cultures of metastatic melanoma cells and eventually in xenograft models for preclinical validation. Since we focused only on FDA approved drugs, successful animal experiments could lead to clinical trials faster than with new drugs. Our results indicate that 4 out of 9 drugs, selected as high scorers in the Connectivity Map analysis of metastatic melanoma, are in fact strong inducers of apoptosis in melanoma cell lines, demonstrating that these unrelated drugs may indeed lead to antimelanoma efficacy. Our next plans are to execute the proposed xenograft experiments in mice as soon as we are able to generate more funding to support this project to demonstrate that at least one of these four drugs elicits significant efficacy against melanoma growth and metastasis. Such demonstrated anti-tumor efficacy would be key for advancing a drug for human clinical testing. Especially, if the drug is used for another disease, initiating clinical testing might entail primarily a strong storyline reinforced by convincing animal studies demonstrating *in vivo* anti-tumor efficacy.

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Appendices:
None

Supporting Data:
None